

Hydrolytic breakdown of lactoferricin by lactic acid bacteria

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Abstract Lactoferricin is a 25-amino acid antimicrobial peptide fragment that is liberated by pepsin digestion of lactoferrin present in bovine milk. Along with its antibacterial properties, lactoferricin has also been reported to have immunostimulatory, antiviral, and anticarcinogenic effects. These attributes provide lactoferricin and other natural bioactive peptides with the potential to be functional food ingredients that can be used by the food industry in a variety of applications. At present, commercial uses of these types of compounds are limited by the scarcity of information on their ability to survive food processing environments. We have monitored the degradation of lactoferricin during its incubation with two types of lactic acid bacteria used in the yogurt-making industry, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, with the aim of assessing the stability of this milk protein-derived peptide under simulated yogurt-making conditions. Analysis of the hydrolysis products isolated from these experiments indicates degradation of this peptide near neutral pH by lactic acid bacteria-associated peptidases, the extent of which was influenced by the bacterial strain used. However, the data also showed that compared to other milk-derived bioactive peptides that undergo complete degradation under these conditions, the 25-amino acid lactoferricin is apparently more resistant,

with approximately 50% of the starting material remaining after 4 h of incubation. These findings imply that lactoferricin, as a natural milk protein-derived peptide, has potential applications in the commercial production of yogurt-like fermented dairy products as a multi-functional food ingredient.

Keywords Bioactive peptide · Lactic acid bacteria · Lactoferricin · Lactoferrin · RP-HPLC

Introduction

Milk has been known to have nutritional benefits for many years. It is a nourishing food that serves as the primary source of nutrition for mammals during the early stages of development. Much research has been directed to understanding the individual components that convey these nutritional benefits and properties to milk and milk products. While milk is made up of a variety of components including caseins, lactalbumin, and immunoglobulins, other minor proteins, such as lactoferrin, have been associated with the prevention of infection [22]. Lactoferrin, an iron-binding glycoprotein found in the whey protein fraction of bovine milk, has specifically been shown to have antimicrobial, antioxidative, immunomodulation, iron-absorption, and anticarcinogenic properties [13, 20].

Lactoferrin has a long history of study in dairy food science dating back to 1939, when its isolation from bovine milk as a red protein was first reported [16]. In 1959, it was purified and characterized as an iron-chelating glycoprotein [6]. Ensuing studies showed that apolactoferrin (iron-free lactoferrin) had an antibacterial effect on strains of *Streptococcus mutans* and *Vibrio cholerae*, while later work extended its antimicrobial effect to *S. pneumoniae*,

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Pseudomonas aeruginosa, *Candida albicans* [1], *Actinobacillus actinomycetemcomitans*, and *Escherichia coli* [21]. More recently, lactoferrin has also been shown to inhibit a number of enveloped viruses, including herpes simplex virus 1 and 2, human immunodeficiency virus (HIV), and human cytomegalovirus [12].

Early work on bovine lactoferrin revealed that enzymatic treatment with pepsin releases potent peptide fragments whose antimicrobial activities are far higher than that of the parent protein [19]. Active peptides generated by pepsin digestion were subsequently isolated, purified, and sequenced, leading to the identification of the bactericidal portion of lactoferrin [2]. This sequence analysis revealed a 25-amino acid peptide corresponding to the first 25 residues of whole lactoferrin, subsequently named lactoferricin. Interestingly, this peptide was found not contain any of the motifs that were previously identified as the iron-binding portions of the lactoferrin, implying that the metal-chelating properties of lactoferrin are not responsible for this antimicrobial activity. It was later shown that bacteria treated with lactoferricin show a loss of membrane integrity, providing evidence for its mechanism of action [21].

The commercial application of antimicrobials in food products to limit the growth of food spoilage bacteria has been well established. Natural compounds produced by food-associated entities exhibiting antibacterial properties are especially attractive for use in food products. While lactoferricin is a multi-functional milk protein-derived peptide that has potential applications as an antimicrobial agent in foods, its commercial use to date has been limited. Studies have shown that it is effective against many food-borne pathogens, such as *Listeria monocytogenes* and *E. coli*, under low pH and refrigeration conditions that are characteristic for yogurt storage [9].

Cell-envelope-associated peptidase/protease systems in *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* strains have been reported to degrade other milk protein-derived antimicrobial and antihypertensive peptides [10]. In the study reported here, we focused on the effect of peptidases present in bacterial strains of yogurt starter cultures on lactoferricin, with the aim of exploring the potential use of this peptide as a food preservative in this particular food system.

Materials and methods

Bacterial strains, growth media, and resting cell assays

All bacterial strains used in this study were from an in-house collection. *Streptococcus thermophilus* ST101 and ST109 were deposited in the Agricultural Research Service

Culture Collection, NCAUR-USDA, Peoria, IL (<http://nrri.ncaur.usda.gov>) as NRRL B-59384 and NRRL B-59385, respectively. Strain ST119 was obtained originally as ATCC14485 (American Type Culture Collection, Rockville, MD). The *S. thermophilus* strains were grown in TYL broth [15], whereas *Lactobacillus delbrueckii* ssp. *bulgaricus* (LAB) strains LB6 (NRRL B-59387), LB11 (NRRL B-59388), and LB20 (NRRL B-59390) were grown in deMann, Rogosa and Sharpe medium (MRS; Becton–Dickinson, Sparks, MD). All ST and LB cultures were propagated at 37°C.

The 25-amino acid peptide lactoferricin (95+% purity) was synthesized by EZBiolab (Westfield, IN). The sensitivity of the peptide to putative cell-bound peptidase enzyme activity was tested by using resting cell suspensions of ST and LB strains prepared from mid-exponential phase ($OD_{660} = 0.5$) cultures (20 ml/tube). The cultures were centrifuged at 10,000 g for 15 min at 4°C. Cell pellets were washed twice in 10 mmol l^{-1} phosphate buffer (pH 7.0) and reconstituted in 1 ml 10 mmol l^{-1} phosphate buffer, representing a 20-fold increase in cell concentration. Serially diluted samples plated on MRS agar showed an average plate count of 10^9 cfu ml^{-1} , which was 1 log higher than the average total bacterial count specified for yogurt products [3]. Reaction mixtures (cell suspensions + peptide) were incubated at 37°C.

Reverse phase-high performance liquid chromatography time studies

The 25-mer lactoferricin peptide was used at a concentration of 50 μg ml^{-1} in assays to test its sensitivity to LAB. Aliquots from these assays were removed and analyzed (10 μl) by reverse phase-high performance liquid chromatography (RP-HPLC) carried out on an Agilent 1200 instrument (Agilent Technologies, Amstelveen, The Netherlands) using a Vydac C18 peptide column (5 mm, 4.6 \times 250 mm). The RP-HPLC analyses were run as a flow rate of 1 ml min^{-1} at 25°C, with solvent A consisting of 0.1% trifluoroacetic acid (Sigma–Aldrich, St. Louis, MO) in Millipore water, and solvent B consisting of 0.086% trifluoroacetic acid in acetonitrile (JT Baker, Phillipsburg, NJ).

Analysis was performed using a linear elution gradient of 5–100% solvent B (95 to 0% solvent A) over 50 min for all samples. Absorbance was recorded at 220 nm using Agilent ChemStation HPLC software. Peaks corresponding to peptide degradation products after 4-h incubation times (absorbance at 220 nm) were collected using an Agilent 1200 series fraction collector and analyzed by matrix-assisted laser desorption ionization–time of flight/time of flight mass spectrometry (MALDI-TOF/TOF-MS) employing an Applied Biosystems 4700 Proteomics Analyzer–MALDI-TOF instrument (Applied Biosystems,

Foster City, CA). Mass spectral data were analyzed further using the FindPept tool provided by the ExPASy Proteomics server (www.expasy.ch). The amount of lactoferricin remaining after exposure to nonproliferating lactic acid bacteria was calculated using a standard curve constructed for this peptide by measuring the absorption of stock solutions ($0\text{--}25\text{ }\mu\text{g ml}^{-1}$) at 220 nm.

Results

RP-HPLC analysis of lactoferricin with lactic acid cultures

The effect of the yogurt starter cultures *S. thermophilus* strains ST101, ST108, ST119, and *L. delbrueckii* ssp. *bulgaricus* strains LB6, LB11, and LB20 on the integrity of the 25-mer lactoferricin was evaluated by monitoring the degradation of the peptide at pH 7.0 by RP-HPLC. Analysis of the samples of lactoferricin treated with either LB6 or LB11 gave very similar results. The chromatographs for both samples showed that the peak corresponding to lactoferricin decreased by approximately 50% after 1 h of treatment. Subsequent incubation with these bacterial strains at longer time points between 1 and 4 h did not show any significant further degradation of this peptide (Fig. 1). Conversely, RP-HPLC analysis of lactoferricin samples treated with LB20 at pH 7.0 revealed an almost complete loss of the peak corresponding to the peptide after just 1 h of incubation (Fig. 2).

Incubation of lactoferricin with nonproliferating cells of ST strains at pH 7.0 yielded comparable results. In all three cases (ST101, ST108, and ST119), proteolysis occurred within the first hour, resulting in a loss of almost half of the starting peptide, as evidenced by RP-HPLC analysis (Fig. 3). The RP-HPLC results showed that the peak corresponding to lactoferricin did not decrease any further in

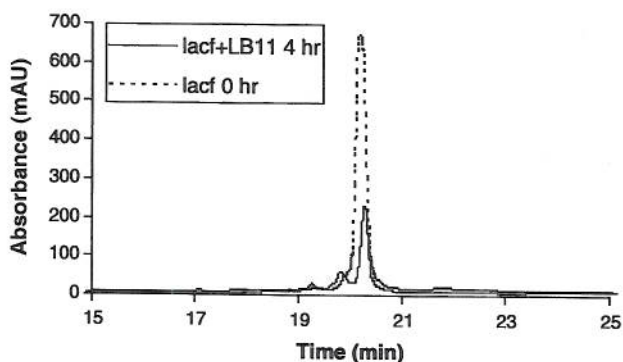


Fig. 1 Lactoferricin (Lacf) incubated with *Lactobacillus delbrueckii* ssp. *bulgaricus* strain LB11 (NRRL B-59388) (LB11) cells after 4 h. Results are also representative of lactoferricin incubated with LB6 (NRRL B-59387) cells for 4 h

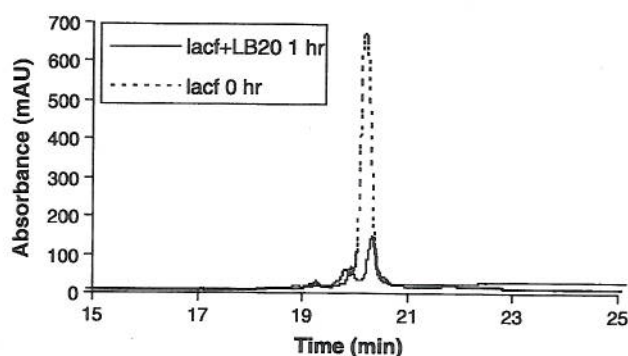


Fig. 2 Reverse phase-high performance liquid chromatography chromatogram (RP-HPLC) of Lacf after incubation with *L. delbrueckii* ssp. *bulgaricus* strain LB20 (NRRL B-59390) cells for 1 h

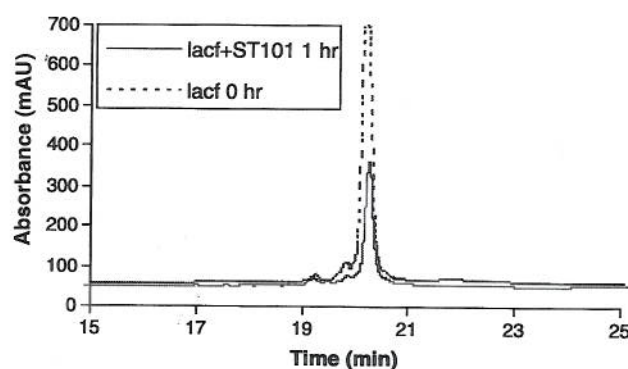


Fig. 3 Lactoferricin (Lacf) after a 1-h incubation with *Streptococcus thermophilus* strain ST101 cells (ST101; NRRL B-59384). Results were similar for Lacf incubated with all ST strains tested

size when the incubation proceeded beyond 1 h, indicating that there was no subsequent degradation. The amounts of lactoferricin lost after exposure to various ST and LB strains for varying lengths of time were calculated and are shown in Table 1.

MALDI-TOF/TOF MS analysis of ST-lactoferricin degradation products

Samples of degradation products were collected during the RP-HPLC analysis and more closely examined by MALDI-TOF/TOF-MS (Fig. 4). Inspection of the mass spectrometric data after 4 h of incubation of lactoferricin with ST101 revealed that a significant amount of proteolysis had occurred. In some cases, the observed masses corresponded to possible cleavage at several sites, and it was not possible to distinguish these fragments without further MS/MS sequence information. The sequence of the 25-amino acid peptide lactoferricin **FKCRRWQWRMKKLGAPSITCVRRAAF** contains a number of basic lysine (K) and arginine (R) residues. These sites are cleavage targets for proteases, such as trypsin, which show a specificity for

Table 1 Hydrolytic breakdown of 25-mer lactoferricin by ST and LB strains at pH 7.0

Strain	Incubation time (h)	Peptide remaining (%)
Control—no bacteria	0	100
ST101	1	54
ST101	4	44
ST108	1	52
ST108	4	45
ST119	1	53
ST119	4	39
LB6	1	39
LB6	4	32
LB11	1	45
LB11	4	34
LB20	1	22
LB20	4	21

ST, *Streptococcus thermophilus* strains; LB, *Lactobacillus delbrueckii* ssp. *bulgaricus* strains

basic amino acids. Inspection of the 4-h incubation mass spectrometric data obtained from ST101 reveals that many of these sites served as cleavage points within the peptide for the ST-associated peptidases. Specifically, cleavage at all three lysine residues at positions 2, 11, and 12 (cleavage at lys12 and arg22 generates a peak at $m/z = 1017.60$ Da, Fig. 4) is observed, as well as proteolysis at the carboxyl end of arginine residues at position 9, 22 and 23. While no cleavage was observed at the C-terminal ends of arginine residues at positions 4 and 5, some proteolysis was observed at the N-terminal ends of arginine 4 (peak at $m/z = 1134.59$ Da, Fig. 4) and arginine 9 (peak at

$m/z = 1118.60$ Da, Fig. 4). Additional masses corresponding with possible random cleavage sites, such as the carboxyl end of cysteine 3 and 20, tryptophan 6 and 8, leucine 13 (peak at $m/z = 1277.70$ Da, Fig. 4), alanine 15, and isoleucine 18, were also observed.

Incubation of lactoferricin with ST108 cells provided degradation products with similar sites of proteolysis. The basic amino acid cleavage sites at the C-terminal end of arginine 4, 5, 9, and 22 are observed in this sample, as well as cleavage at lysine 11. Additionally, the other sites of proteolysis that are observed here are consistent with those in the sample from the incubation mixture with ST101 cells, with the additional cutting observed at the carboxyl side of threonine 19. In the case of the incubation of lactoferricin with ST119 cells, similar results were obtained, with the majority of observed proteolysis taking place at sites within the sequence C-terminal to lysine (2, 11, and 12) or arginine (5, 9, 22, and 23) residues. Masses corresponding to additional cleavage at tryptophan (6 and 8), glutamine (7), methionine (10), leucine (13), alanine (15), serine (17), threonine (19), cysteine (20) and valine (21) residues were also observed in the mass spectrometric data.

Mass spectrometric analysis of LB-lactoferricin degradation products

The isolation of samples by RP-HPLC following the incubation of lactoferricin samples with cells of strain LB6 revealed similar results with respect to cleavage near arginine residues to those obtained from the samples with the ST cultures. Masses corresponding to fragments cleaved at arginines 5 and 21 were observed as well as peptide fragments resulting from cleavage at cysteine 3, glutamine 7, leucine

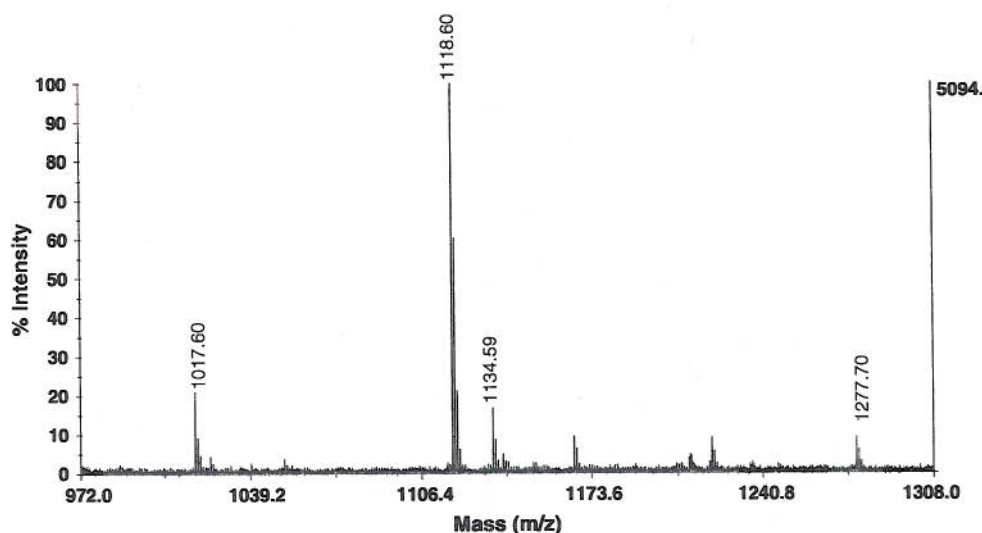


Fig. 4 Example of mass spectrometric data obtained from analysis of RP-HPLC fractions. This spectrum corresponds to degradation products that were visible following a 4-h incubation of Lacf with ST101 cells

13, glycine 14, alanine 15 and 24, proline 16, serine 17, and valine 21. No cleavage around the lysine residues was observed. The incubation of lactoferricin with LB11 cells, however, resulted in a number of peaks corresponding to almost thorough cleavage at every amino acid residue in the sequence. Masses corresponding to proteolysis at residues 1, 2, 3, 5, 6, 7, 10, 11, 12, 13, 14, 15, 17, 22, and 24 were observed, suggesting random enzymatic digestion. In contrast, products of lactoferricin incubated with cells of strain LB20 mostly showed cleavage at the anticipated locations, near arginine 4, 5, 9, and 23 and lysine 2 and 11. Some additional fragments corresponding to cleavage at cysteine 3 and 20, tryptophan 6, leucine 13, glycine 14, and threonine 19 were also observed.

Discussion

Lactoferricin is a peptide of interest that has been studied for a number of years. Because of its antibacterial, immunostimulating, antioxidative, and antiviral properties, it has a number of potential commercial applications [8, 20]. Harnessing these possible benefits could be achieved if a successful mode of delivery could be developed, such as incorporation into everyday consumer products. This has prompted an interest in determining the feasibility for introducing lactoferricin, a milk protein-derived bioactive compound, into dairy products, such as yogurt, as a method for passing onto consumers its nutritional and health benefits.

There has been a significant amount of work aimed at pinpointing the exact amino acid residues required for the bioactivity of lactoferricin [11]. Two tryptophan residues at positions 6 and 8 have been found to be necessary for antimicrobial activity [17]. Primary amino acid sequence changes have also been incorporated into truncated lactoferricin peptides (consisting of residues 1–25) in an effort to increase the potency of this peptide. The substitution of residues 1, 4, and 11 with arginine and residue 13 with a tyrosine led to a greater than tenfold improvement in antimicrobial activity of this peptide against *E. coli* and *S. aureus* [18]. It has also been shown that truncating the lactoferricin peptide even further—to an 11-mer peptide consisting of residues 4–14—generates a potent antimicrobial compound with decreased hemolytic activity relative to the parent peptide [7].

The results presented here show that incubation of lactoferricin with strains of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* yogurt fermentation bacteria leads to considerable proteolysis. This is not surprising due to the well-documented presence of cell-associated peptidases and proteases in both types of yogurt-fermenting bacteria [4, 5, 14]. It is interesting to note, however, that in the cases of

both ST (strains 101, 108, and 119) and LB (strains 6 and 11) cultures, complete degradation of the 25-mer lactoferricin peptide did not occur: approximately half of the peptide was lost after 1 h of incubation with these particular strains, and even after 4 h of incubation at pH 7.0, half of the initial peptide still remained. Total degradation of lactoferricin was only observed for LB20, with approximately 80% of the peptide found to be lost after a 1-h incubation. Mass spectrometric analysis of the proteolytic fragments of the peptide revealed predominantly random cleavage of the peptide with some proteolysis taking place at lysine and arginine residues.

Earlier studies have shown that utilizing the truncated 11-mer version of lactoferricin in these types of investigations also results in complete, efficient proteolysis at pH 7.0 by both ST and LB cultures, revealing its limited utility as an antimicrobial supplement in yogurt if added at the start of the fermentation process [10]. The full-length (25-mer) peptide, while still subject to proteolysis, has been shown here to be much more robust at shorter (up to 4 h) time periods than its truncated derivative. The disulfide bond in the 25-mer peptide between cysteines at positions 3 and 20 possibly provides some protection against proteolysis. These cysteine residues are not present in the 11-mer peptide, which may explain the differences in degradation observed with the two substrates.

One of the major challenges when incorporating bio-functional or supplemental products into foods is to ensure that the added compound remains intact and retains efficacy throughout the processing events involved in the manufacturing processes, which may include high or low temperatures, changes in pH environments, among others. These properties are also important in the case of fermented milk products that incorporate and utilize bacterial cultures in addition to the customary processing requirements. The results of this study indicate that it may be possible to utilize full-length lactoferricin in yogurt products due to the latter's relatively increased ability to remain stable during processing. Incorporating non-natural amino acids that are resistant to proteolysis into the lactoferricin sequence may ultimately reveal a derivative that can fully survive yogurt fermentation processes and allow for the commercial use of these types of milk protein-derived peptides.

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